


## Male age is associated with sperm DNA/chromatin integrity


Anupama Deenadayal Mettler<sup>a</sup>, Mirudhubashini Govindarajan<sup>b</sup>, Sapna Srinivas<sup>a</sup>, Sridurga Mithraprabhu<sup>b,c</sup>, Donald Evenson<sup>d</sup> and Tara Mahendran<sup>e</sup> 

<sup>a</sup>Infertility Institute and Research Centre, Secunderabad, India; <sup>b</sup>Womens Center, Coimbatore, India; <sup>c</sup>Australian Centre for Blood Diseases, Alfred Hospital – Monash University, Melbourne, Australia; <sup>d</sup>SCSA Diagnostics, Inc, Brookings, SD, USA; <sup>e</sup>Andrology Center, Coimbatore, India

### ABSTRACT

The diagnosis of sperm DNA integrity is increasingly recognized as being crucial to inform the clinical course in infertile couples. An internationally accepted sperm DNA fragmentation assay that determines the proportion of sperm and degree of broken sperm nuclear DNA with recognised clinical thresholds for identifying men at risk of infertility is the Sperm Chromatin Structure Assay (SCSA<sup>®</sup>). In this study, SCSA<sup>®</sup> test was utilised to evaluate the relevance of male age on sperm DNA quality in total of 6881 males of Indian origin. Analysis of proportions of DNA fragmentation index (%DFI) and high DNA stainability (%HDS) was performed based on four groups (<35, 35–40, 40–45, and >45 years of age). The impact of increasing male age on %DFI revealed that males >45 years of age had the highest %DFI and lowest %HDS compared to all other age groups ( $p < .001$ ). This study is the largest population study and first of its kind in India that utilises SCSA<sup>®</sup> to assess the relevance of %DFI and %HDS to increasing age with potentially important implications for the choice of clinical course based on age and sperm quality of infertile males in India.

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### Introduction

Infertility is defined as the failure to achieve a clinical pregnancy after a year or more of regular unprotected intercourse when the female partner is <35 years of age or after 6 months of regular unprotected intercourse when the female partner is >35 years of age [1,2]. Recent trends of delayed parenthood highlight the detrimental effects of ageing on a couple's fertility. More specifically, it appears that in females in India, ovaries appear to age earlier, had lower implantation and ongoing pregnancy rates when compared to Caucasian women, indicating that ethnicity is an independent predictor of IVF/ICSI outcomes [3,4]. The current focus still remains on the age of the female partner, even though it is now widely accepted that the male factor is responsible for up to 40% of cases of couple infertility. Amongst the multitude of factors responsible for male infertility, compromised sperm quality is of utmost significance. The diagnosis of sperm DNA integrity is increasingly recognized as being crucial to inform the clinical course in infertile couples. Intact sperm DNA is essential for optimal

embryo growth and development. The conventional parameters of semen analysis are insufficient for evaluation of reproductive potential.

The Sperm Chromatin Structure Assay (SCSA<sup>®</sup>) is the only sperm DNA fragmentation (SDF) assay that simultaneously determines the percentage of sperm with fragmented (broken) DNA, the degree of DNA damage and the extent of uncondensed chromatin (HDS) [5,6]. DNA fragmentation index (DFI) scores indicate the likelihood of sperm contributing to infertility issues with SCSA<sup>®</sup> providing accepted clinical thresholds for placing men at risk of infertility and being more predictive than conventional semen analyses [6–8]. Specifically, DFI is known to increase in an age dependant manner in men as demonstrated by a number of studies in both Caucasian and non-Caucasian males [9–14]. High DNA stainability (HDS) is a measure of the abnormal lack of condensation of the sperm chromatin likely caused by abnormal methylation, which can be identified with the increased levels of histones [15,16]. Similar to sperm with increased DFI, semen samples with high levels of sperm with partially uncondensed chromatin have an

increased probability of early embryo cessation of growth and miscarriage [17–19]. SCSA<sup>®</sup> is the only SDF test that can simultaneously measure %DFI and %HDS and is a powerful tool to provide critical information to inform clinical course and the choice of assisted reproductive technology (ART).

Limited number of large scale population studies has been conducted for evaluation of sperm quality in males presenting with infertility, specifically in the Indian sub-continent [20–23]. The present study is the largest and first of its kind to utilize the internationally accepted SCSA<sup>®</sup> test in a population of males in India to assess the effect of male age on %DFI and %HDS. The study will assess the role of increasing male age on %DFI and %HDS to determine if this age dependent decline in sperm DNA quality also affects Indian males, as observed in other populations and to define the thresholds for %DFI and %HDS in this population of sub-fertile men so as to inform the best clinical course based on sperm DNA quality.

## Materials and methods

### Study population and participants

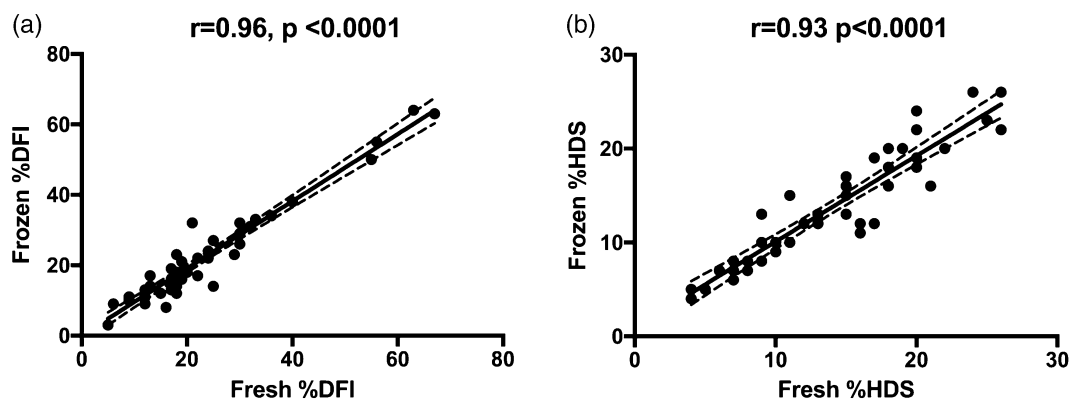
Samples from 6881 male patients who attended infertility clinics in India between 2013 and 2016 were examined in this study. Semen samples were collected following informed consent and samples were analysed fresh or frozen/thawed at the Andrology Center (Coimbatore, India). Correlation of age to %DFI and %HDS was performed retrospectively. All patients were assessed for their Serology status for Viral/Bacterial infections including HIV-1 and -2, HBsAg, HCV, and VDRL test before undergoing the SCSA<sup>®</sup> test. A detailed analysis of medical information of patients comprising past surgical history, recent medical illness/medical conditions like fever/diabetes, blood pressure, cholesterol, high BMI was obtained through patient information forms obtained only for a small cohort of patients and therefore was not analysed in this study. Patients were also evaluated for their lifestyle and occupational factors such as history of cigarette smoking, alcohol consumption, diabetes, viral/bacterial infection within past 4 weeks, fever within past six months, varicocele (existing condition/ repaired), cardiac history, neural or nephritic disease, medication, exposure to chemotherapy/radiation or family history of any genetic disease. Clinical outcome of pregnancy was not available; therefore, assessment of the impact of %DFI and %HDS on fertility was not performed.

### Semen collection

Prior to sample collection, the patients were asked to follow an abstinence regime spanning 24–48 h. The semen samples were collected by masturbation into a sterile wide mouthed calibrated container. After liquefaction of 1 h at room temperature, 200–500  $\mu$ L of the raw semen were aliquoted into cryovials without cryoprotectant and flash frozen in liquid nitrogen. The samples were then transported to the centralised testing lab at Andrology Centre (Coimbatore, India) in liquid nitrogen dry shippers ( $-196^{\circ}\text{C}$ ) or in dry ice ( $-80^{\circ}\text{C}$ ).

### SCSA<sup>®</sup> test protocol

Individual semen samples, stored in liquid nitrogen tanks ( $-196^{\circ}\text{C}$ ) were thawed in a  $37^{\circ}\text{C}$  water bath and then immediately placed on crushed ice. As previously described [24], an aliquot of raw semen was transferred to TNE buffer (0.01 M Tris-HCl:0.15 M NaCl:1 mM EDTA, pH 7.4) at  $4^{\circ}\text{C}$  to a final concentration of  $\sim 1\text{--}2 \times 10^6$  sperm/mL. A total of 200  $\mu$ L of this sperm suspension was admixed with 400  $\mu$ L of a solution containing 0.08 N HCl:0.15 M NaCl:0.1% Triton X at  $4^{\circ}\text{C}$ . Importantly, the HCl was diluted from commercial 2.0 N HCl. After 30 s, the sperm were stained by adding 1.2 mL of staining solution containing 6  $\mu$ g/mL acridine orange (AO, chromatographically purified, Polysciences, Inc., Warrington, PA): 0.2 M  $\text{Na}_2\text{PO}_4$ :0.1 M citric acid (pH 6.0):1 mM EDTA:0.15 M NaCl; so that the AO/DNA-P molar ratio was  $\geq 2$  [25]. The acid/AO stained sample was placed in a FACS CALIBUR<sup>™</sup> flow cytometer (BD Biosciences, San Jose, CA) sample chamber and sample flow was initiated to bring the sheath flow and sample flow to equilibrium by 2 min. Subsequently, 5000 sperm were analyzed at an event rate of 100–250 events/s. If the event rate was above 250 events/s, a new sample was prepared to ensure full equilibrium between the AO dye and sperm. Each sample was analysed in duplicate and these replicates of the data were utilised for the percentage of sperm with measurably increased red fluorescence (sperm with fragmented DNA) and those with HDS using proprietary SCSAsoft<sup>®</sup>. The results were generated as a clinical report expressed as %DFI and %HDS. Correlation between measures of %DFI on 57 aliquots of frozen/thawed semen samples at SCSA Diagnostics (Brookings, SD) and the Andrology Lab (Coimbatore, India) was:  $R^2 = 0.9812$  [26].



**Figure 1.** No differences between frozen and fresh samples. (A) Correlation of %DFI between fresh and frozen samples ( $r=0.96$ ,  $p<.0001$ ). (B) Correlation of %HDS between fresh and frozen samples ( $r=0.93$ ,  $p<.0001$ ).

### Statistical analysis

All statistical analyses were performed using GraphPad Prism 6.0f (La Jolla, CA). Detailed descriptions are provided within the results. A  $p$  value of  $\leq .05$  was considered statistically significant and only significant differences are reported.

### Results

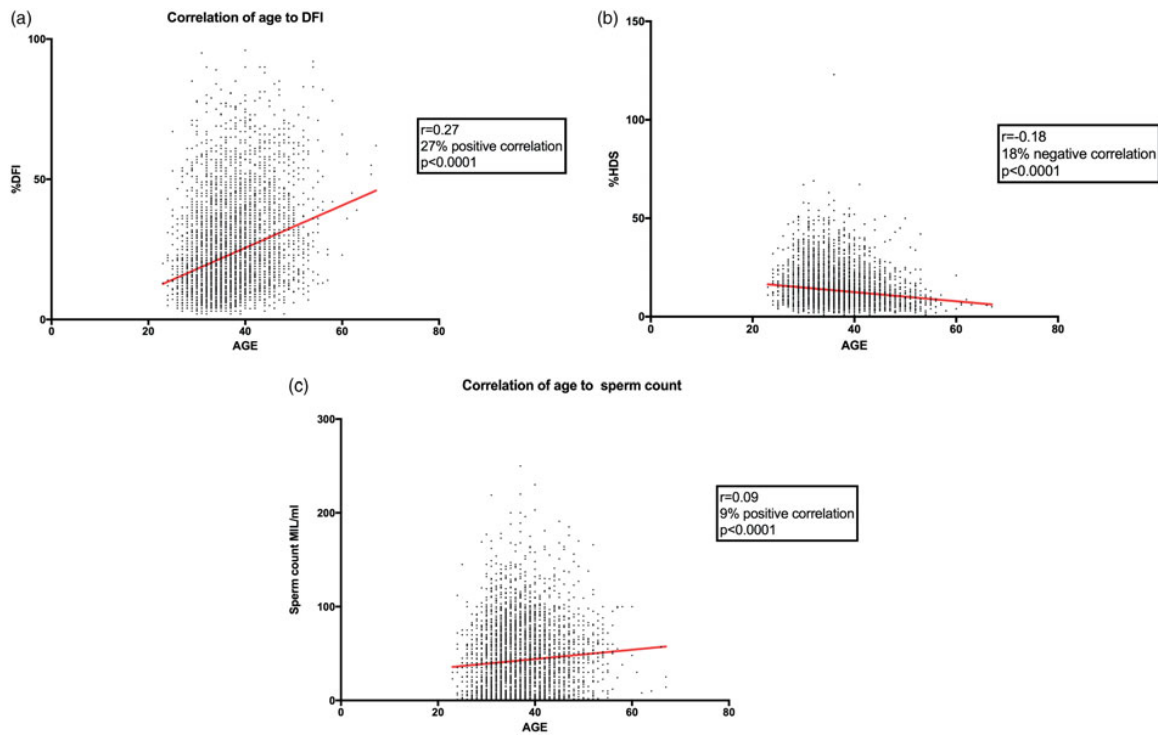
#### Frozen or fresh samples can be utilised for SCSA assay

A majority of samples acquired for this study were frozen samples sourced from several parts of India. Previously published literature suggests that DNA fragmentation is affected by cryopreservation [27,28]. Therefore, comparison of fresh and frozen samples was undertaken to ascertain the usability of frozen samples. SCSA<sup>®</sup> was performed for  $n=44$  patients for whom analysis was performed 1 h after sample collection or following cryopreservation. The %DFI and %HDS were found to be similar in patients in fresh compared to frozen samples with a 96% concordance for %DFI and 93% concordance for %HDS in the data (Figure 1(A,B), Spearman's  $r$  correlation test,  $p<.0001$ ).

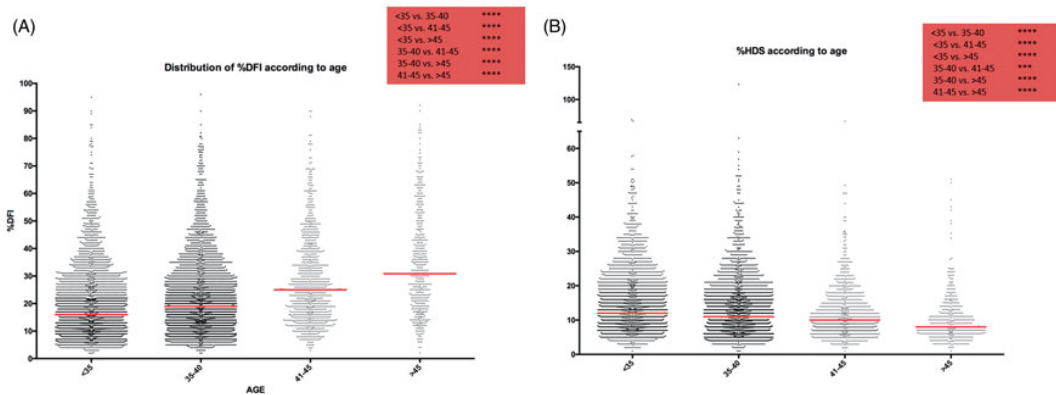
#### Increasing male age is significantly associated with higher DFI and lower HDS

A total of 6881 men were assessed for %DFI and %HDS using SCSA<sup>®</sup>. The age range was between 23 and 61 years, with the highest number of patients ( $n=601$ ) at 35 years of age (data not shown). Patients were divided into four groups based on their age – group 1 – patients  $<35$  years of age, group 2 – patients between 35 and 40 years of age, group 3 – patients between 40 and 45 years of age, and group 4 – patients  $>45$  years

of age. Correlation of age with %DFI, %HDS, and total sperm count was performed to identify if correlations to age exist. The %DFI from all patients indicated a weak positive but a significant correlation with age ( $r=0.27$ ,  $p<.0001$ , Figure 2(A), Spearman's  $r$  correlation test). Similarly, correlation of age with %HDS showed a weak negative and significant correlation in patients ( $r=-0.18$ ,  $p<.0001$ , Figure 2(B), Spearman's  $r$ ). Age to total sperm count showed a weak positive correlation of 9% ( $r=0.09$ ,  $p<.0001$ , Figure 2(C), Spearman's  $r$ ). Patients were then segregated based on age into four groups ( $<35$ , 35–40, 41–45, and  $>45$ ) and the levels of %DFI and %HDS were assessed. The median %DFI levels of each of these categories were calculated and were found to be significantly elevated with increasing male age (Figure 3(A), the Kruskal–Wallis test,  $p<.0001$ ). Men  $<35$  years of age had a median %DFI of 16%, 35–40 had 19%, 41–45 with 25% and the  $>45$  age group had a median %DFI of 30.8. Additionally, 17% (1181) of the patients had  $\geq 30\%$  DFI with the median age of patients in this group as 38 years. The proportion of HDS sperm based on increasing age was also analysed and this was found to be inversely proportional to age, i.e. older patients had less %HDS (Figure 3(B), the Kruskal–Wallis test,  $p<.001$  at least). The median HDS levels at  $<35$ , 35–40, 41–45, and  $>45$  were 12, 11, 10, and 8, respectively, with all groups significantly different to each other ( $p<.0001$ ). %DFI was correlated with sperm count based on age revealing a relatively stronger negative correlation in males  $\leq 45$  years of age compared to  $>45$  age group (Figure 4(A–D)). %HDS correlation to sperm count indicated that a consistent negative correlation between  $r=-0.34$  and  $-0.28$  was present in all age groups (Figure 5(A–D)). Furthermore, information regarding profession was available for 425 patients that included four different types of



**Figure 2.** Correlations of all patients %DFI, %HDS and sperm count by age. (A) Correlation of all patients with age to %DFI indicates a weak positive correlation ( $r=0.27$ ,  $p<.0001$ ). (B) Correlation of age to %HDS indicates a weak negative correlation ( $r=-0.18$ ,  $p<.0001$ ). (C) Correlation of age to sperm count shows a weak positive correlation ( $r=0.09$ ,  $p<.0001$ ).



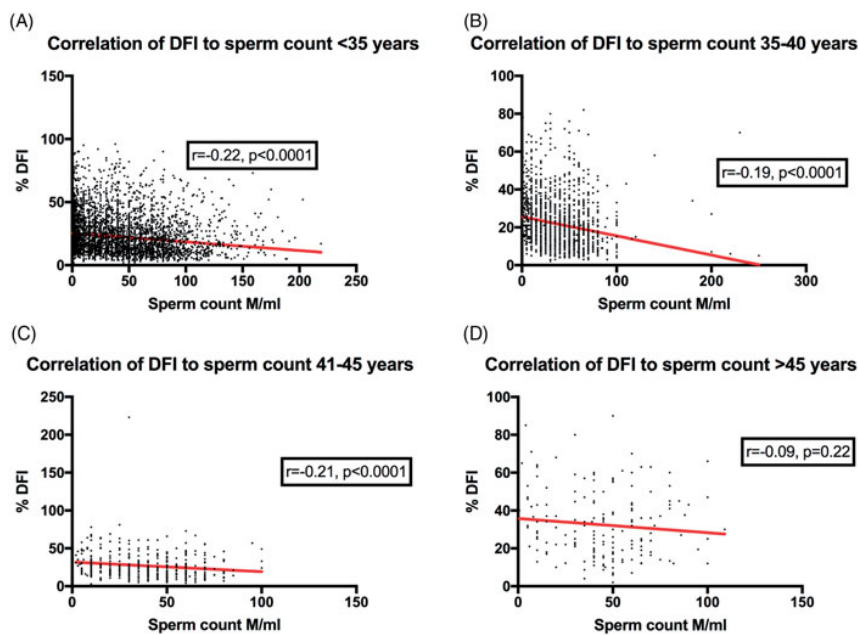
**Figure 3.** Increasing male age is significantly associated with higher DFI and lower HDS. (A) Column graph of %DFI in four age categories <35, 35–40, 40–45, and >45 showing increasing levels of %DFI with increasing age with the median %DFI line in black ( $***p<.0001$ ). (B) Column graph of %HDS in four age categories showing decreasing levels of %HDS with increasing age with the median %HDS line in black ( $***p<.001$ ;  $****p<.0001$ ).

profession: teachers, business, farming/agriculture, and information technology. There were no significant differences in %DFI or %HDS based on the profession (data not shown).

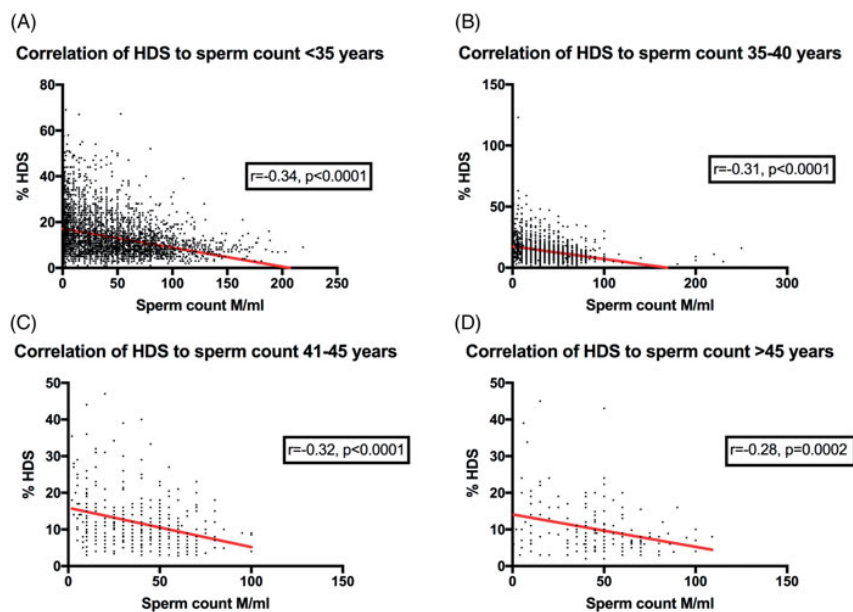
### Discussion

Infertility clinics are currently faced with couples opting delayed parenthood, declining semen parameters, and increased SDF. Therefore, SDF assessment is

becoming more relevant due to poor reproductive outcomes, lifestyle factors, and recurrent pregnancy losses [29]. The value of SDF testing and the clinical importance has also been acknowledged in the latest American and European Urology Associations (AUA and EUA, respectively) and the American Society of Reproductive Medicine (ASRM) Practice Committee [30,31]. As age appears to play a significant role in SDF, in our study we have assessed the importance



**Figure 4.** Negative correlation of DFI to sperm count. (A) Correlation of sperm count to %DFI in males <35 years of age with  $r = -0.22, p < .0001$  (B) Correlation of sperm count to %DFI in males between 35 and 40 years of age with  $r = -0.19, p < .0001$ . (C) Correlation of sperm count to %DFI in males between 40 and 45 years of age with  $r = -0.21, p < .0001$ . (D) Correlation of sperm count to %DFI in males >45 years of age with  $r = -0.09, p = .22$ .



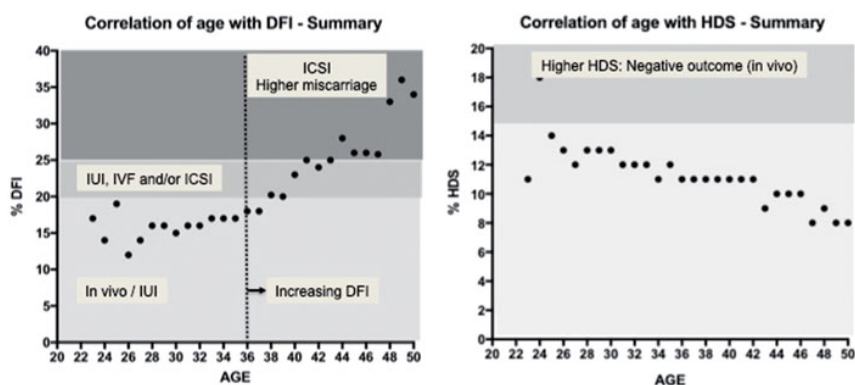
**Figure 5.** Decreasing correlation between %HDS and sperm count with age. (A) Correlation of sperm count to %HDS in males <35 years of age with  $r = -0.34, p < .0001$ . (B) Correlation of sperm count to %HDS in males between 35 and 40 years of age with  $r = -0.31, p < .0001$ . (C) Correlation of sperm count to %HDS in males between 40 and 45 years of age with  $r = -0.32, p < .0001$ . (D) Correlation of sperm count to %HDS in males >45 years of age with  $r = -0.28, p = .001$ .

of increasing male age on %DFI and %HDS in males from India to reveal significant associations. It must be noted that this is the first study that has assessed %HDS and %DFI in a large Indian population and has revealed that older men have significantly smaller

%HDS and higher %DFI in accordance with the hypothesis that sperm from older men are more damaged and harbour an immature chromatin.

SCSA<sup>®</sup> testing can be performed on both fresh and cryopreserved samples. Given that the semen samples





**Figure 6.** Summary of DFI correlation with age. Increasing rate of %DFI is evident in males. The suggested clinical work up based on %DFI levels is presented here. The age point in which the median %DFI from <20% to an increased rate at above >20% DFI is at age 36 (dotted line). Infertility clinic couples with <20% DFI could opt for *in vivo* or IUI procedures. With >20% DFI, an ART procedure is suggested with levels >25% requiring possibly several ICSI cycles. Higher miscarriage rates are also evident in sperm with higher %DFI. HDS levels above 15% are known to have negative natural pregnancy outcomes; however, while in our data a decreasing HDS level is observed with increasing age, the consequence of this in selecting an appropriate ART procedure is unclear as studies are currently based on HDS levels and ICSI/IVF outcomes have differing results.

are received cryopreserved from different parts of India, differences in %DFI and %HDS between frozen and fresh samples from the same patient were analysed. Sperm DNA damage proportions were comparable between frozen and fresh samples, indicating that either of these sample types could be utilised for assessing sperm DNA damage. This is consistent with what is previously published [6,32]. Furthermore, one of the main concerns regarding collection and processing of semen samples from different locations is inter-lab variability. All the samples were processed at a single location, so the data are comparable.

Correlation of the age of all patients to %DFI, %HDS and sperm count did not show strong correlations; one possible reason for this is because of the extensive age range and therefore samples were demarcated into four age groups to identify further differences. Assessment of age-wise %DFI in patients showed that patients <35 years of age had 16% DFI and >40 years of age had >25% DFI. With increasing male age, there is a steady increase in the %DFI and decrease in %HDS, indicating that an increase in age is a critical factor for sperm DNA damage. These DFI findings in our study support observations from previous studies that have associated male age with DNA damage [14,33–35]. Furthermore, younger men with lesser sperm count had a significantly higher probability of harbouring increased %DFI. While %DFI and impact of male age has been reported previously, relatively lesser number of studies have provided information about %HDS levels. In our study, levels of HDS steadily declined with increasing male age. Additionally, it was also observed that a significant

negative correlation was associated with the sperm count in all age groups, as reported previously [15]. Sperm decondensation is indicative of nuclear immaturity arising from abnormal tertiary structure leading to faulty compaction. Therefore, reduced HDS could be an impact of slower rate of spermatogenesis and increased disulphide bonding [16]. Abnormal nuclear decondensation is also known to contribute to repeated IVF failures with developmental arrests at the pronuclear stage [36]. HDS values also appear to play a critical role in sperm head morphology with incomplete sperm chromatin condensation being a contributory factor to sperm head defects [37]. A study done by Wyrobek et al. found a slight negative correlation of HDS with age in unadjusted analysis; however, when adjusted for sexual abstinence, this correlation was absent. This could be due to the small population size and selection of samples from a non-clinical setting compared to our study of patients reporting to a fertility clinic [35]. Proportions of DFI and HDS were also assessed based on the profession and there were no changes. Further information is currently being collected to assess more patients and parameters.

The %DFI is a critical factor to determine the clinical course as suggested previously [5,26,38]. Four useful clinical thresholds for %DFI and male factor infertility have been defined. (1) Twenty percent DFI has no negative impact, (2) 20–25% DFI begins with negative consequences and in particular if one or more classical semen parameters are significantly abnormal, (3) >25% DFI portends poor results for both natural and intra-uterine insemination (IUI), and (4) >40% DFI results in low probability of pregnancy

and an increased risk of spontaneous miscarriages providing the necessary demarcations for choosing the DFI threshold above which ICSI should be performed [5,26,38]. An HDS >15% has a negative impact in natural pregnancies [17]; however, more studies with IVF/ICSI procedures need to be performed to determine the impact of HDS levels on ART outcomes [39,40]. In our study, clinical outcomes of fertility treatment were not available and therefore this was not assessed. However, ongoing studies within our facility have incorporated clinical information and this aspect will be assessed for future publications.

A summary of the SDF levels according to age as revealed by our study with a suggested clinical work-up for infertile couples based on previously published studies that define clinical course for %DFI and %HDS is presented in Figure 6. It is clear that ageing has a significant impact on semen quality and fertility in India. These changes contribute to reduced fecundity and increased miscarriage rates. The assessment of sperm DNA structure is important and necessary in evaluation of male infertility, more so in advanced paternal age. The clinical utility of SDF testing has been established and the study of sperm DNA structure is mandatory in infertility evaluation in cases of previous ART failures and recurrent miscarriages. Our results establish that paternal age is another important clinical indication for SDF analysis, which has been generally neglected thus far. However, it must be noted that SDF and nuclear decondensation are independent processes and each value must be considered independent of the other in treatment management.

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### Disclosure statement

The authors declare no conflict of interest.

### Notes on contributors

*Concept and design:* ADM, MG, DE, and TM were responsible for concept and design of the study. *Administrative support:* ADM, MG, SS, SM, DE, and TM were all involved in administrative support. *Provision of study materials and patients:* ADM, MG, and SS were responsible for providing study materials and patients. *Collection and assembly of data:* ADM, SS, SM, and TM were responsible for collection and assembly of data. *Data analysis and interpretation:* SM and TM were

involved in data analysis and study interpretation. *Manuscript writing:* TM and SM drafted the manuscript and all authors were involved in the critical review and re-drafting of the final version of the manuscript. *Final approval of manuscript:* All authors approved the final version of the manuscript.

### ORCID

Tara Mahendran  <http://orcid.org/0000-0002-9422-7040>

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